Fig. 1

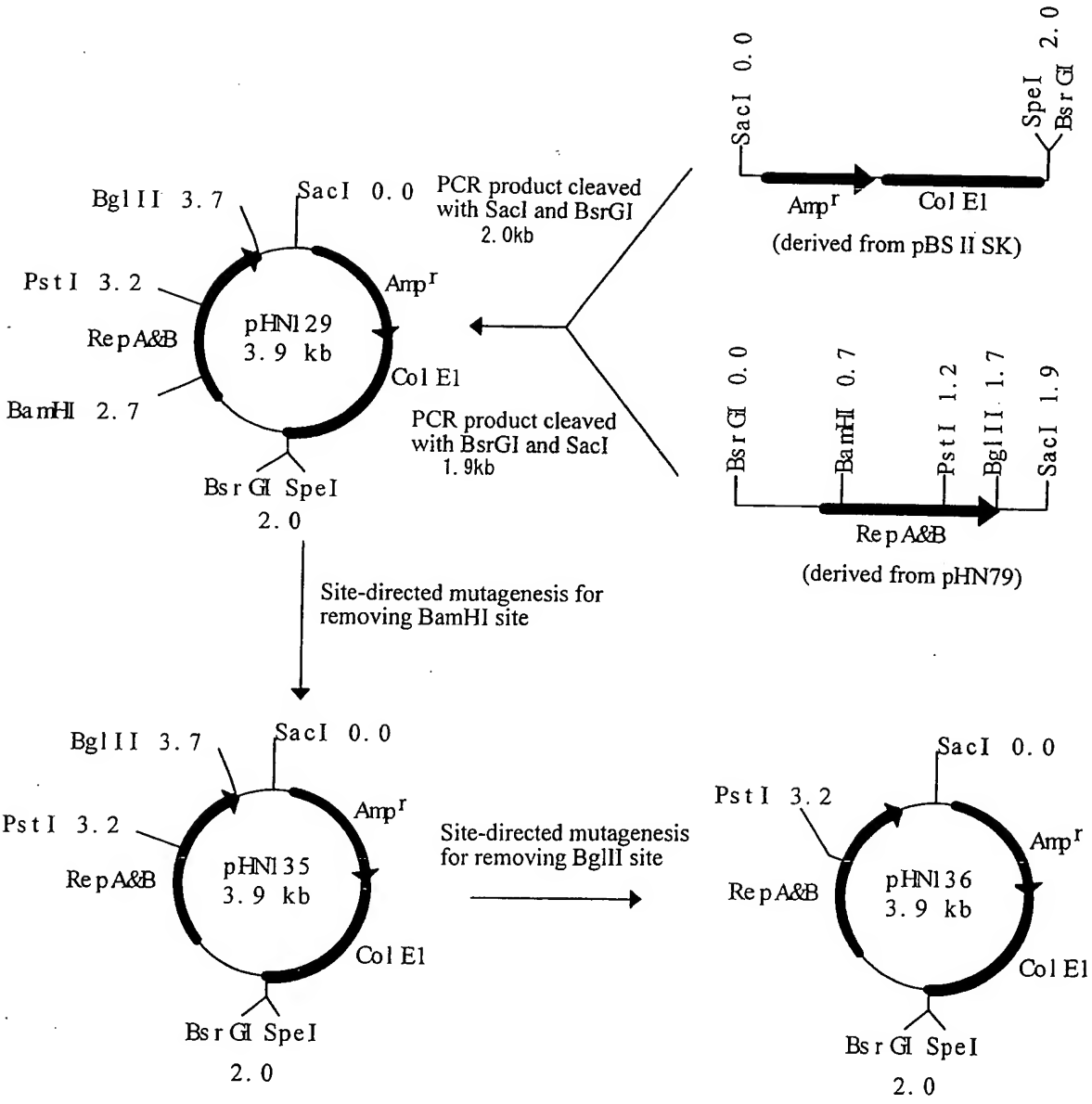

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Fig. 2

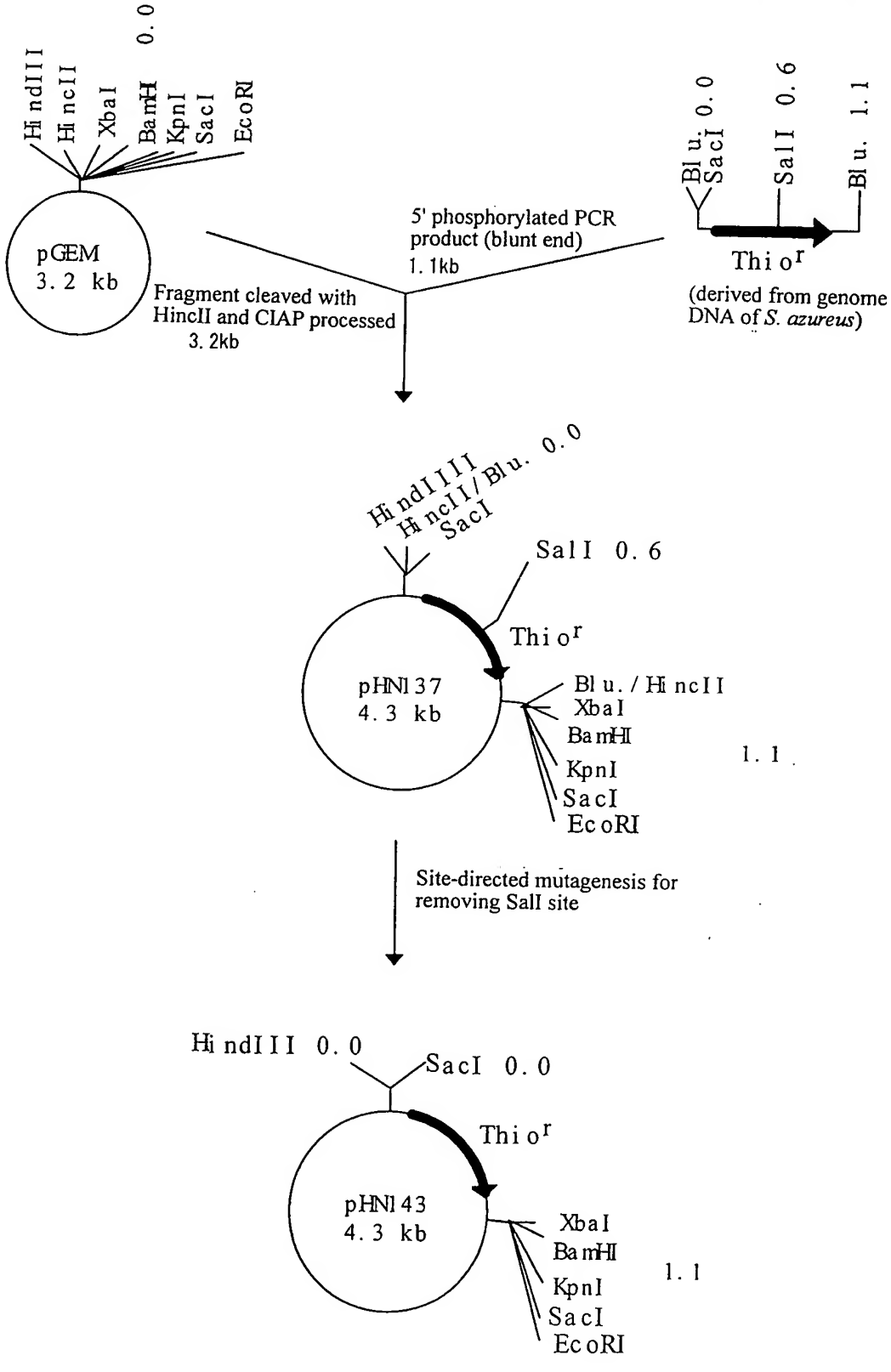
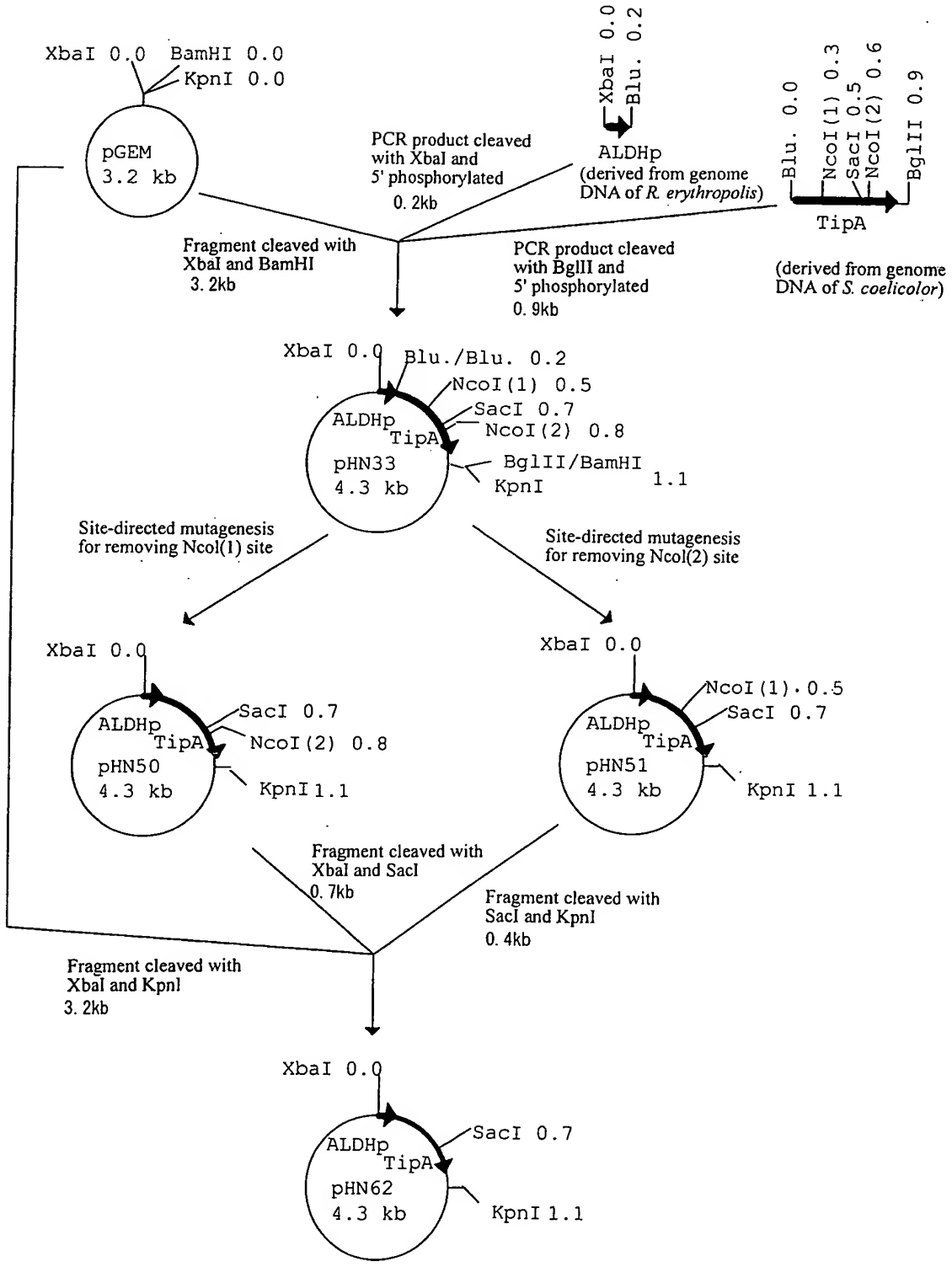

HindIII
HincII
XbaI
BamHI
KpnI
SacI
EcoRI
0.0
pGEM
3.2 kb
Fragment cleaved with
HincII and CIAP processed
3.2kb
5' phosphorylated PCR
product (blunt end)
1.1kb
Blu.
SacI
0.0
SalI 0.6
Blu. 1.1
Thio^r
(derived from genome
DNA of S. azureus)
HindIII
HincII/Blu.
SacI
0.0
SalI 0.6
Thio^r
pHN137
4.3 kb
Blu./HincII
XbaI
BamHI
KpnI
SacI
EcoRI
1.1
Site-directed mutagenesis for
removing SalI site
HindIII 0.0
SacI 0.0
Thio^r
pHN143
4.3 kb
XbaI
BamHI
KpnI
SacI
EcoRI
1.1

Fig. 3


XbaI 0.0
BamHI 0.0
KpnI 0.0
pGEM
3.2 kb
XbaI 0.0
Blu. 0.2
ALDHp
(derived from genome
DNA of R. erythropolis)
PCR product cleaved
with XbaI and
5' phosphorylated
0. 2kb
Blu. 0.0
NcoI(1) 0.3
SacI 0.5
NcoI(2) 0.6
BglII 0.9
TipA
(derived from genome
DNA of S. coelicolor)
Fragment cleaved with
XbaI and BamHI
3. 2kb
PCR product cleaved
with BglII and
5' phosphorylated
0. 9kb
XbaI 0.0
Blu./Blu. 0.2
NcoI(1) 0.5
SacI 0.7
NcoI(2) 0.8
ALDHp
TipA
pHN33
4.3 kb
BglII/BamHI
KpnI
1.1
Site-directed mutagenesis
for removing NcoI(1) site
Site-directed mutagenesis
for removing NcoI(2) site
XbaI 0.0
SacI 0.7
ALDHp
TipA
NcoI(2) 0.8
pHN50
4.3 kb
KpnI 1.1
XbaI 0.0
NcoI(1) 0.5
SacI 0.7
ALDHp
TipA
pHN51
4.3 kb
KpnI 1.1
Fragment cleaved with
XbaI and SacI
0. 7kb
Fragment cleaved with
SacI and KpnI
0. 4kb
Fragment cleaved with
XbaI and KpnI
3. 2kb
XbaI 0.0
SacI 0.7
ALDHp
TipA
pHN62
4.3 kb
KpnI 1.1

10/52415

Fig. 4

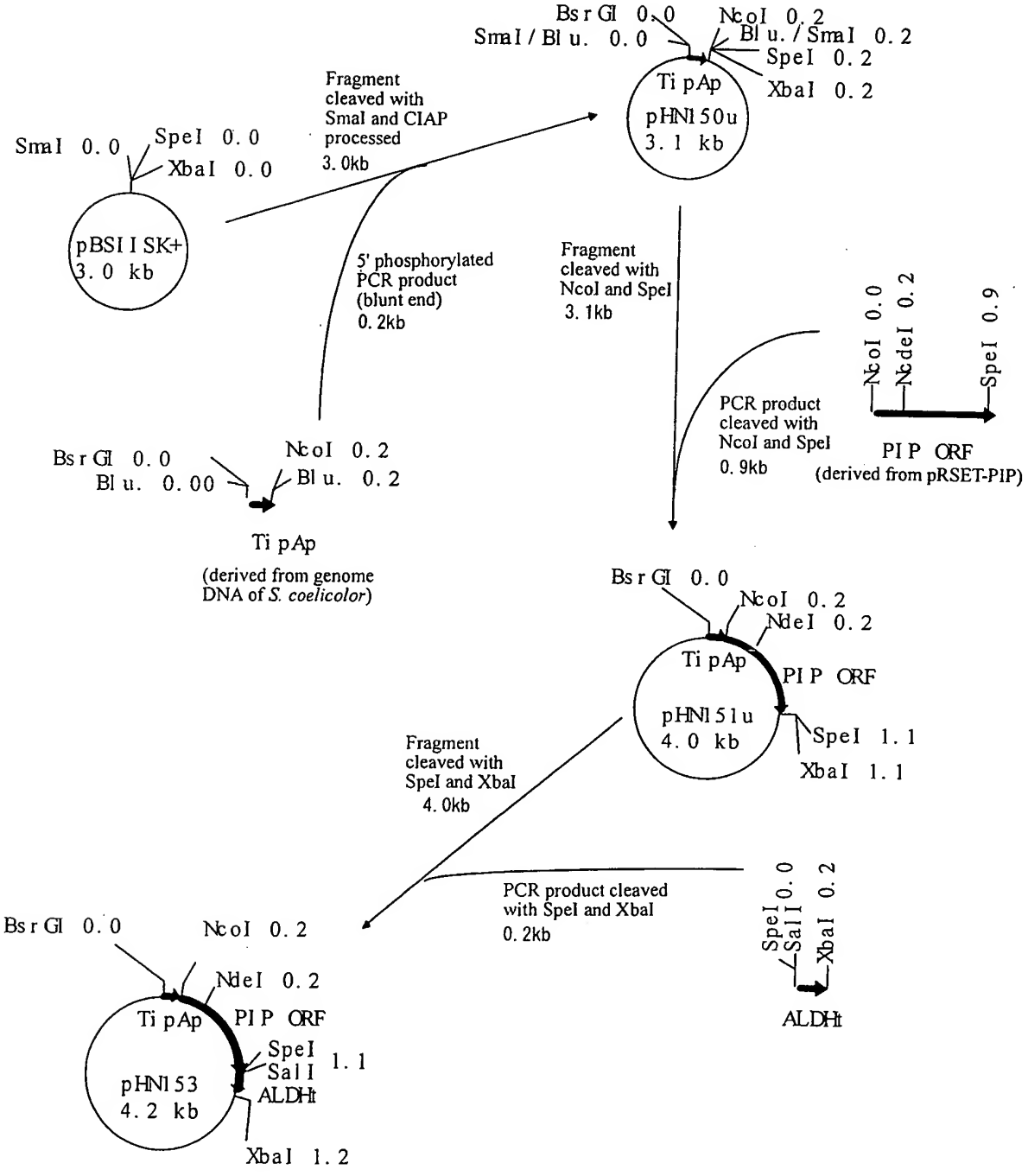


Fig. 5

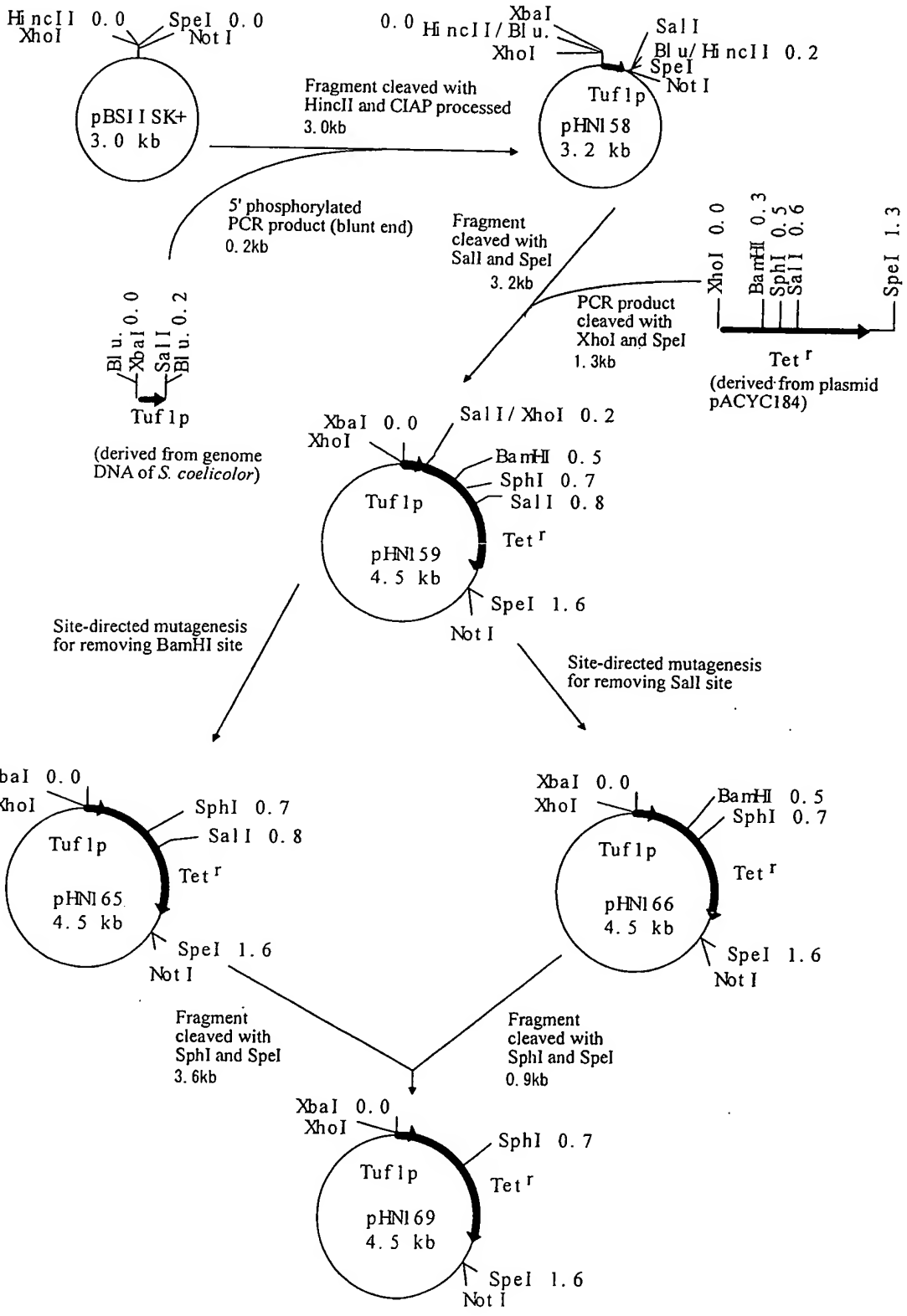

HincII 0.0
XhoI
SpeI 0.0
Not I
pBSII SK+
3.0 kb
0.0 HincII/Blu.
XbaI
XhoI
SalI
Blu/HincII 0.2
SpeI
Not I
Tuf1p
pHN158
3.2 kb
Fragment cleaved with
HincII and CIAP processed
3.0kb
5' phosphorylated
PCR product (blunt end)
0.2kb
Fragment
cleaved with
SalI and SpeI
3.2kb
PCR product
cleaved with
XhoI and SpeI
1.3kb
Blu. 0.0
XbaI
SalI
Blu. 0.2
Tuf1p
(derived from genome
DNA of S. coelicolor)
XhoI 0.0
BamHI 0.3
SphI 0.5
SalI 0.6
SpeI 1.3
Tet r
(derived from plasmid
pACYC184)
XbaI 0.0
XhoI
SalI/XhoI 0.2
BamHI 0.5
SphI 0.7
SalI 0.8
Tuf1p
Tet r
pHN159
4.5 kb
SpeI 1.6
Not I
Site-directed mutagenesis
for removing BamHI site
Site-directed mutagenesis
for removing SalI site
XbaI 0.0
XhoI
SphI 0.7
SalI 0.8
Tuf1p
Tet r
pHN165
4.5 kb
SpeI 1.6
Not I
XbaI 0.0
XhoI
BamHI 0.5
SphI 0.7
Tuf1p
Tet r
pHN166
4.5 kb
SpeI 1.6
Not I
Fragment
cleaved with
SphI and SpeI
3.6kb
Fragment
cleaved with
SphI and SpeI
0.9kb
XbaI 0.0
XhoI
SphI 0.7
Tuf1p
Tet r
pHN169
4.5 kb
SpeI 1.6
Not I

Fig. 6

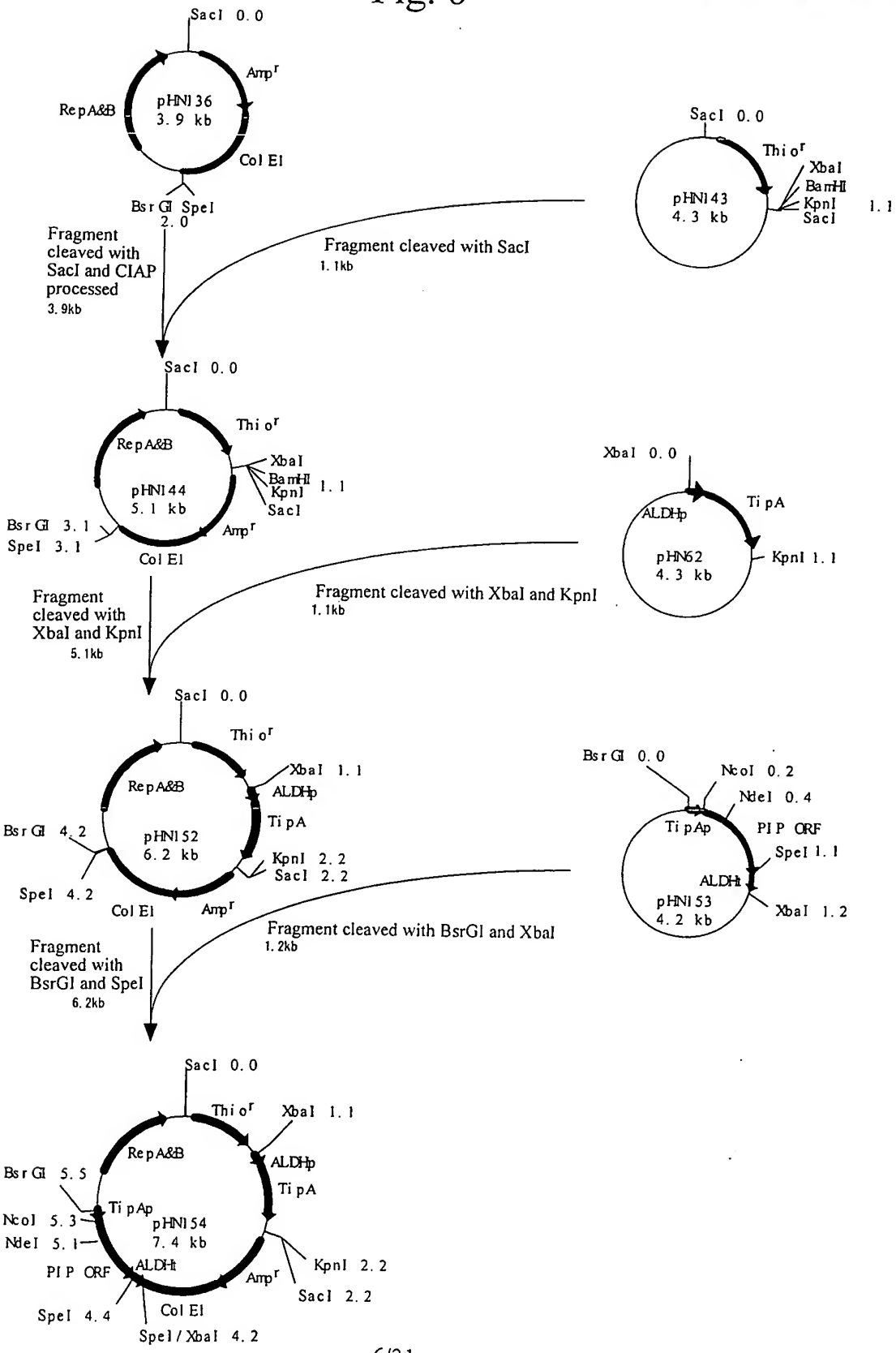
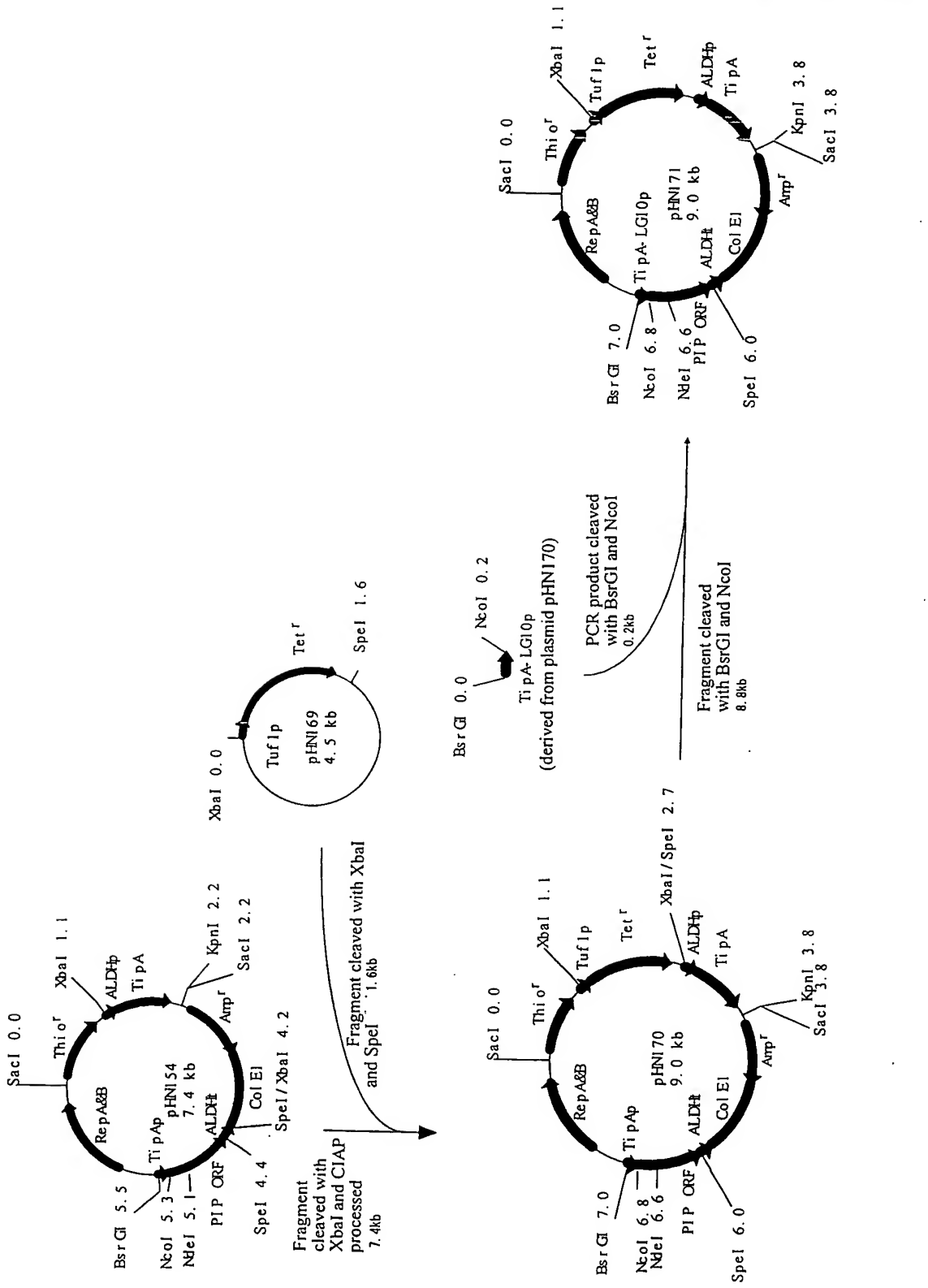

SacI 0.0
Amp^r
RepA&B
pHN136
3.9 kb
ColEI
BsrGI SpeI
2.0
Fragment
cleaved with
SacI and CIAP
processed
3.9kb
Fragment cleaved with SacI
1.1kb
SacI 0.0
Thio^r
XbaI
BamHI
KpnI
SacI
pHN143
4.3 kb
1.1
SacI 0.0
Thio^r
RepA&B
XbaI
BamHI
KpnI 1.1
SacI
pHN144
5.1 kb
BsrGI 3.1
SpeI 3.1
Amp^r
ColEI
Fragment
cleaved with
XbaI and KpnI
5.1kb
Fragment cleaved with XbaI and KpnI
1.1kb
XbaI 0.0
TipA
ALDHp
pHN62
4.3 kb
KpnI 1.1
SacI 0.0
Thio^r
XbaI 1.1
RepA&B
ALDHp
TipA
pHN152
6.2 kb
BsrGI 4.2
SpeI 4.2
KpnI 2.2
SacI 2.2
ColEI
Amp^r
Fragment
cleaved with
BsrGI and SpeI
6.2kb
Fragment cleaved with BsrGI and XbaI
1.2kb
BsrGI 0.0
NcoI 0.2
NdeI 0.4
TipAp
PIP ORF
SpeI 1.1
ALDHt
pHN153
4.2 kb
XbaI 1.2
SacI 0.0
Thio^r
XbaI 1.1
RepA&B
ALDHp
TipA
BsrGI 5.5
TipAp
NcoI 5.3
NdeI 5.1
pHN154
7.4 kb
PIP ORF
ALDHt
Amp^r
KpnI 2.2
SacI 2.2
SpeI 4.4
ColEI
SpeI/XbaI 4.2

Fig. 6


SacI 0.0
Thio^r
XbaI 1.1
ALDHp
TipA
KpnI 2.2
SacI 2.2
Amp^r
ColE1
SpeI/XbaI 4.2
ALDHt
TipAp
pHN154
7.4 kb
RepA&B
BsrGI 5.5
NcoI 5.3
NdeI 5.1
PIP ORF
SpeI 4.4
Fragment cleaved with XbaI and SpeI 1.6kb
Fragment cleaved with XbaI and CIAP processed 7.4kb
XbaI 0.0
Tuf1p
pHN169
4.5 kb
Tet^r
SpeI 1.6
BsrGI 0.0
NcoI 0.2
TipA-LGI0p
(derived from plasmid pHN170)
PCR product cleaved with BsrGI and NcoI
0.2kb
Fragment cleaved with BsrGI and NcoI
8.8kb
SacI 0.0
Thio^r
XbaI 1.1
Tuf1p
Tet^r
XbaI/SpeI 2.7
ALDHp
TipA
KpnI 3.8
SacI 3.8
Amp^r
ColE1
ALDHt
TipAp
pHN170
9.0 kb
RepA&B
BsrGI 7.0
NcoI 6.8
NdeI 6.6
PIP ORF
SpeI 6.0
SacI 0.0
Thio^r
XbaI 1.1
Tuf1p
Tet^r
ALDHp
TipA
KpnI 3.8
SacI 3.8
Amp^r
ColE1
ALDHt
TipA-LGI0p
pHN171
9.0 kb
RepA&B
BsrGI 7.0
NcoI 6.8
NdeI 6.6
PIP ORF
SpeI 6.0

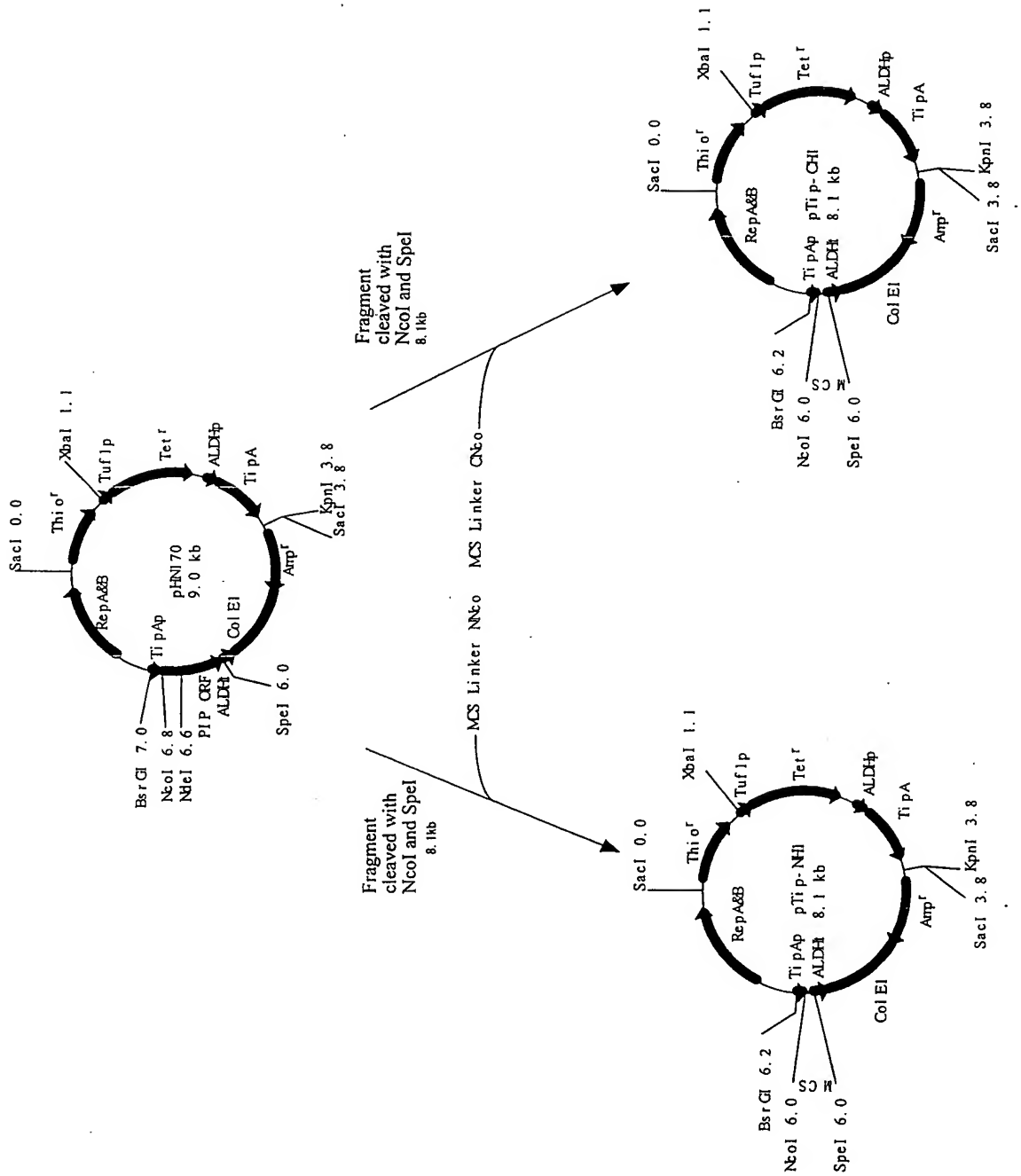

SacI 0.0
Thio^r
XbaI 1.1
TufIp
Tet^r
ALDHp
TipA
KpnI 3.8
SacI 3.8
Amp^r
ColEI
SpeI 6.0
NcoI 6.0
BsrGI 6.2
MCS
RepA&B
TipAp
ALDHt
pHN170
9.0 kb
PIP ORF
NdeI 6.6
NcoI 6.8
BsrGI 7.0
pTip-CHI
8.1 kb
pTip-NHI
8.1 kb
MCS Linker CNco
MCS Linker NNco
Fragment
cleaved with
NcoI and SpeI
8.1kb

Fig. 7

Fig. 7

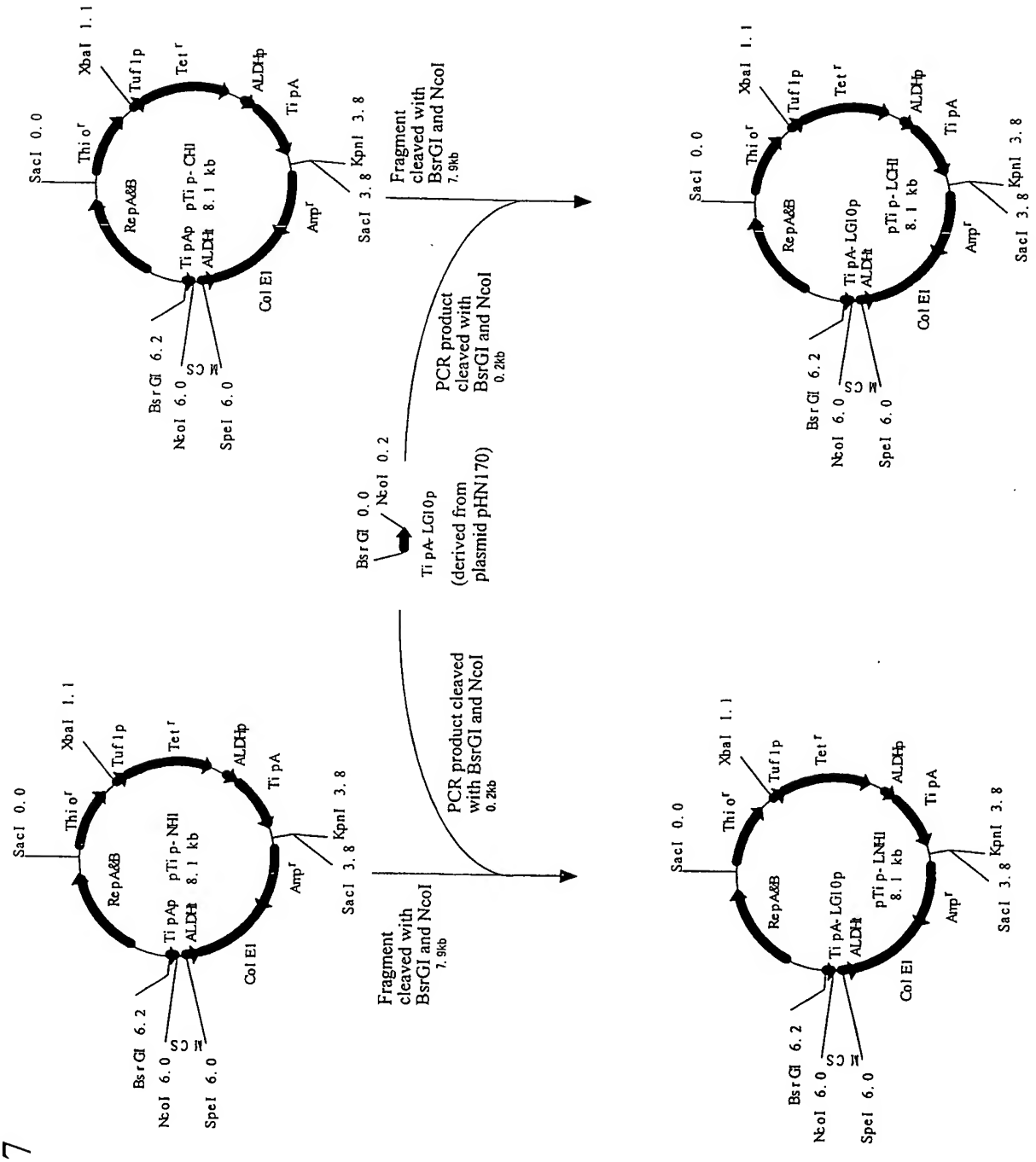
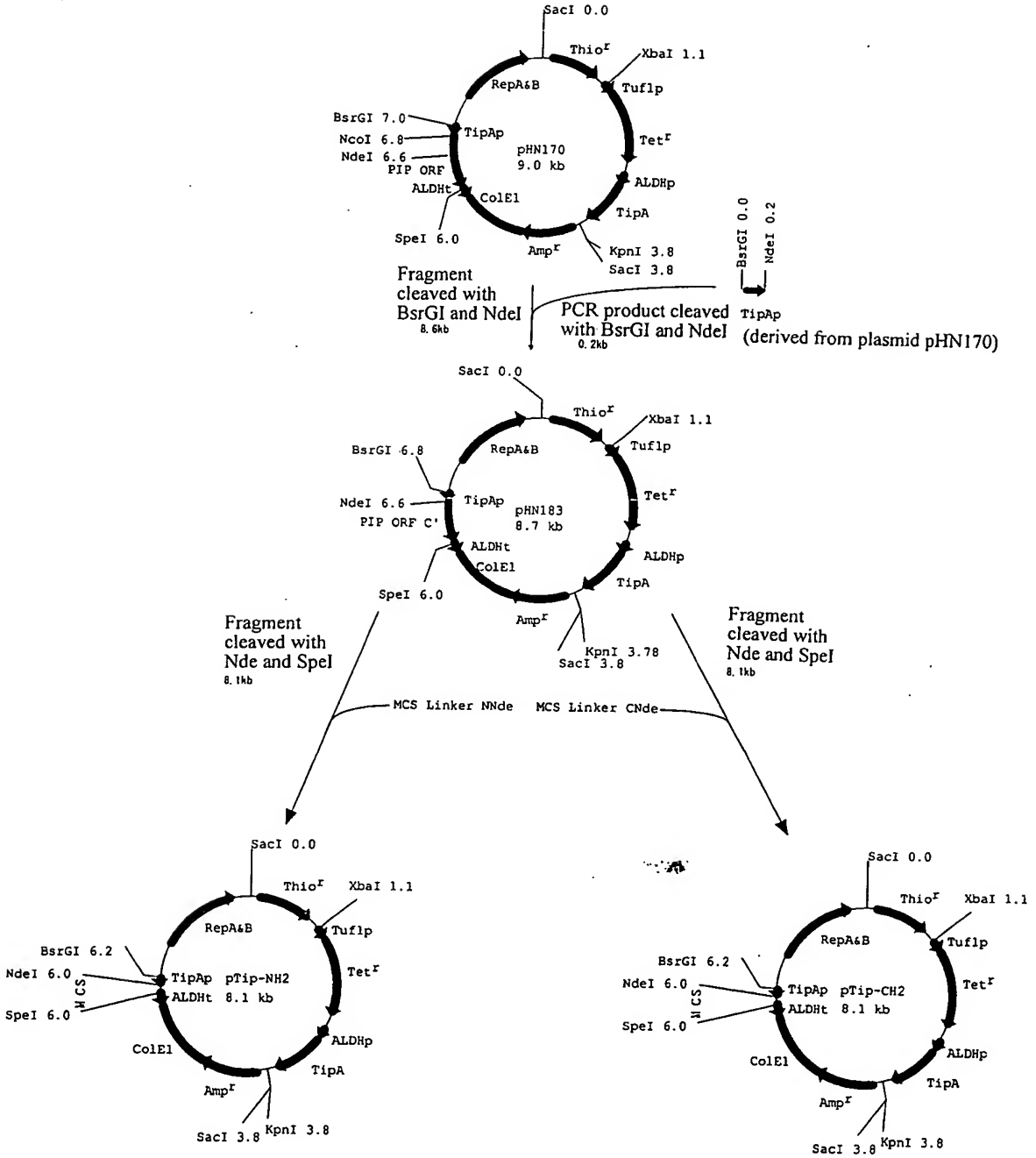

SacI 0.0
Thio^r
XbaI 1.1
TufIp
Tet^r
ALDHp
TipA
KpnI 3.8
Amp^r
SacI 3.8
RepA&B
pTip-CHI
8.1 kb
TipAp
ALDHt
ColEI
BsrGI 6.2
NcoI 6.0
SpeI 6.0
Fragment cleaved with BsrGI and NcoI
7.9kb
BsrGI 0.0
NcoI 0.2
TipA-LGI0p
(derived from plasmid pHN170)
PCR product cleaved with BsrGI and NcoI
0.2kb
pTip-LCHI
pTip-NHI
pTip-LNHI
TipA-LGI0p

Fig. 8


SacI 0.0
Thio^r
XbaI 1.1
RepA&B
TufIp
BsrGI 7.0
TipAp
NcoI 6.8
NdeI 6.6
PIP ORF
pHN170
9.0 kb
Tet^r
ALDHp
ALDHt
ColE1
TipA
SpeI 6.0
Amp^r
KpnI 3.8
SacI 3.8
BsrGI 0.0
NdeI 0.2
Fragment cleaved with BsrGI and NdeI
8.6kb
PCR product cleaved with BsrGI and NdeI
0.2kb
TipAp
(derived from plasmid pHN170)
BsrGI 6.8
NdeI 6.6
PIP ORF C'
pHN183
8.7 kb
KpnI 3.78
Fragment cleaved with Nde and SpeI
8.1kb
MCS Linker NNde
MCS Linker CNde
BsrGI 6.2
NdeI 6.0
MCS N
SpeI 6.0
pTip-NH2
ALDHt 8.1 kb
pTip-CH2
MCS C

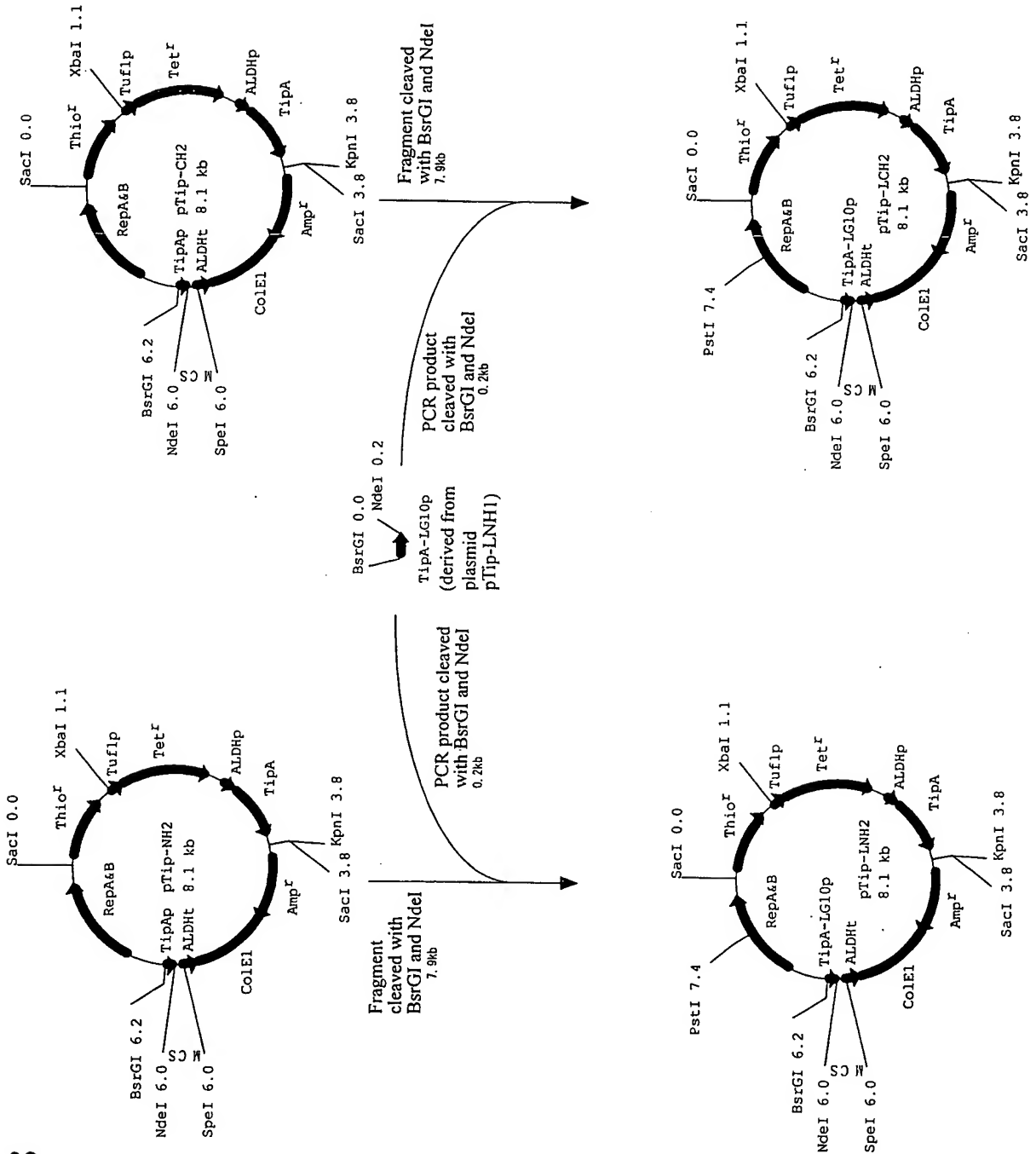
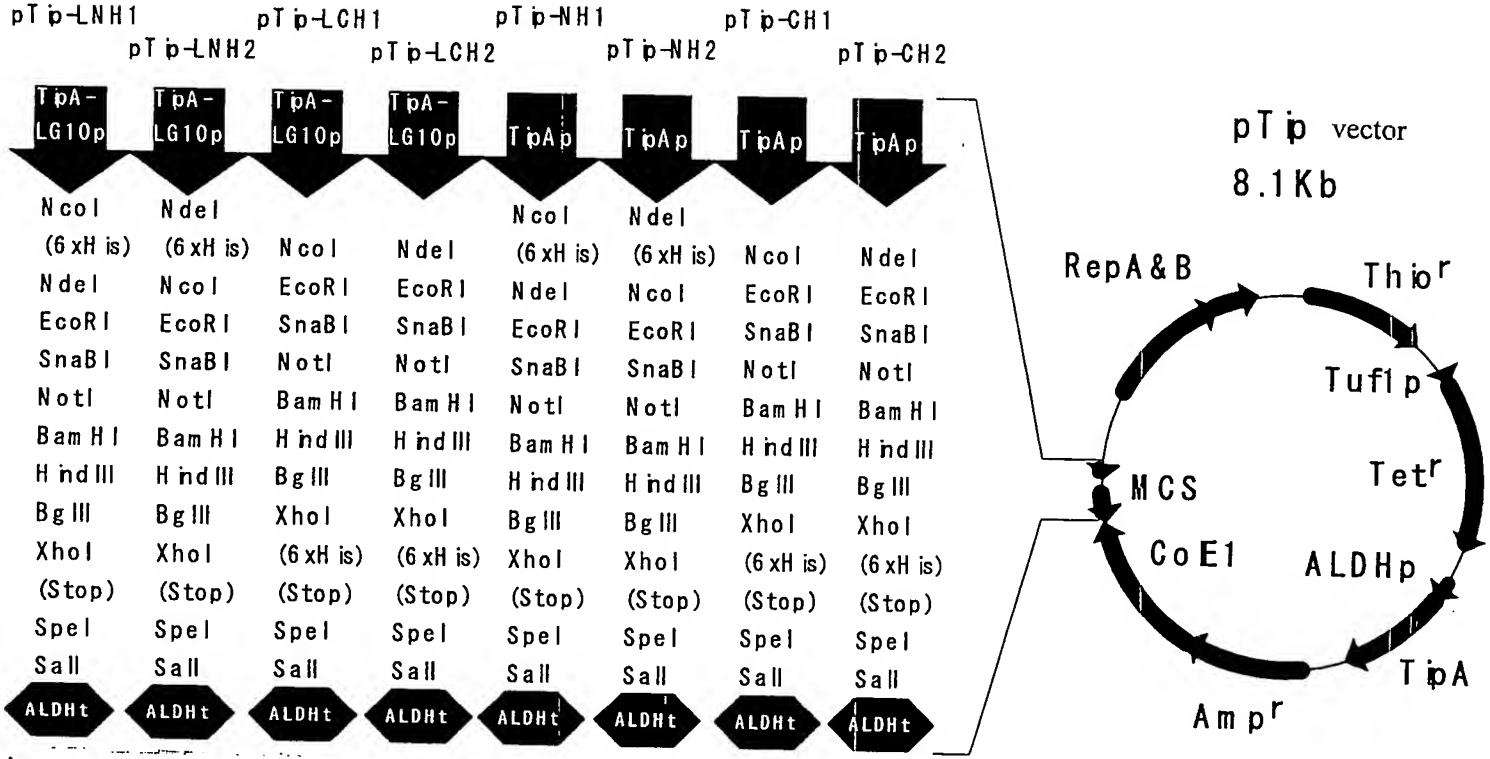

SacI 0.0
Thio^r
XbaI 1.1
TufIp
Tet^r
ALDHp
TipA
KpnI 3.8
SacI 3.8
Amp^r
ColE1
RepA&B
TipAp
pTip-CH2
8.1 kb
ALDHt
BsrGI 6.2
NdeI 6.0
SpeI 6.0
M CS
Fragment cleaved with BsrGI and NdeI
7.9kb
PCR product cleaved with BsrGI and NdeI
0.2kb
BsrGI 0.0
NdeI 0.2
TipA-LG10p
(derived from plasmid pTip-LNH1)
PstI 7.4
pTip-LCH2
pTip-NH2
pTip-LNH2
TipA-LG10p

Fig. 8

Fig. 9a



Thiostrepton induction system

Thio^r= confers thiostrepton resistance to *R.erythropolis*

ALDHp = promoter which constitutively produces TipA protein

TipA = encodes a TipA protein

TipAp = TipA promoter

TipA-LG10p = improved TipA promoter

ALDHt = transcription termination sequence

Regions necessary for the autonomous replication of a plasmid

ColE1 = for *E. coli*

RepA&B = for *R.erythropolis*

Antibiotic resistance marker

Tuf1p-Tet^r = transformation marker for *R.erythropolis*

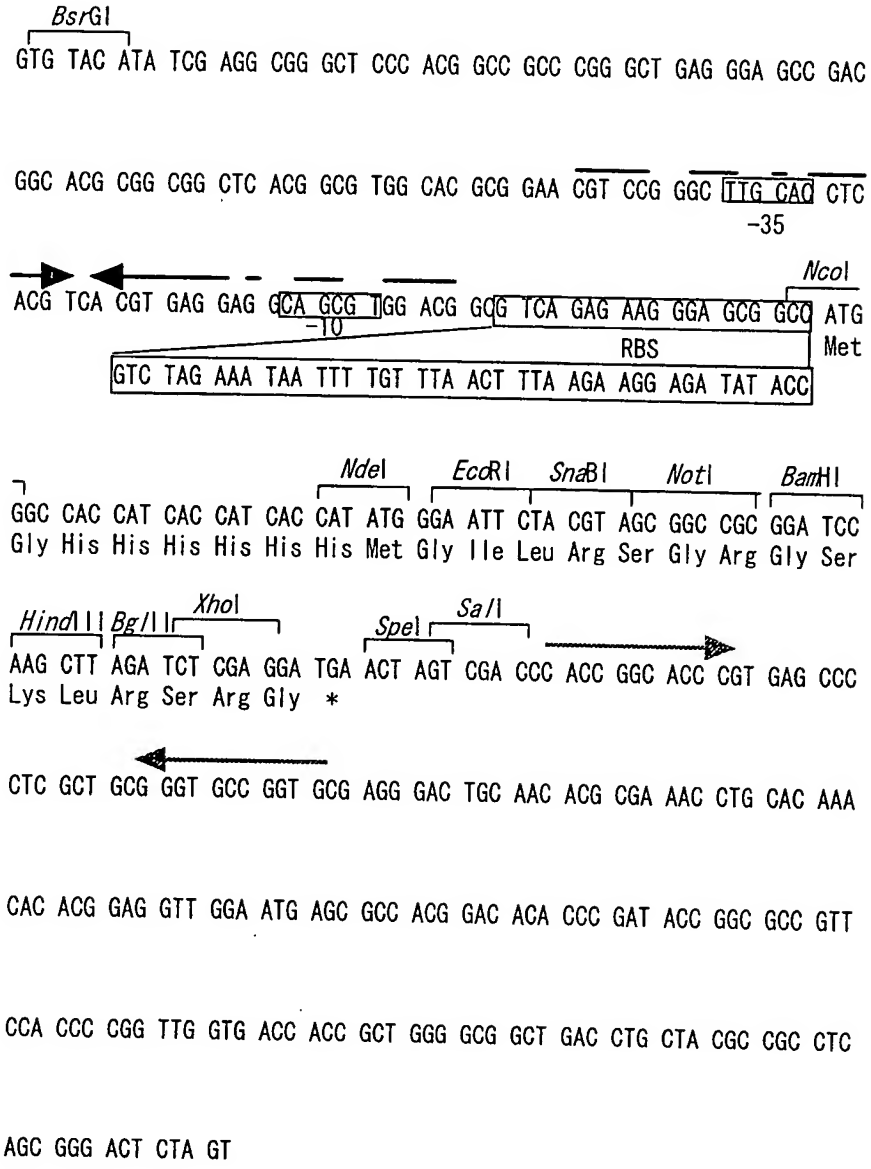
Amp^r = transformation marker for *E. coli*

Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232

10/524193

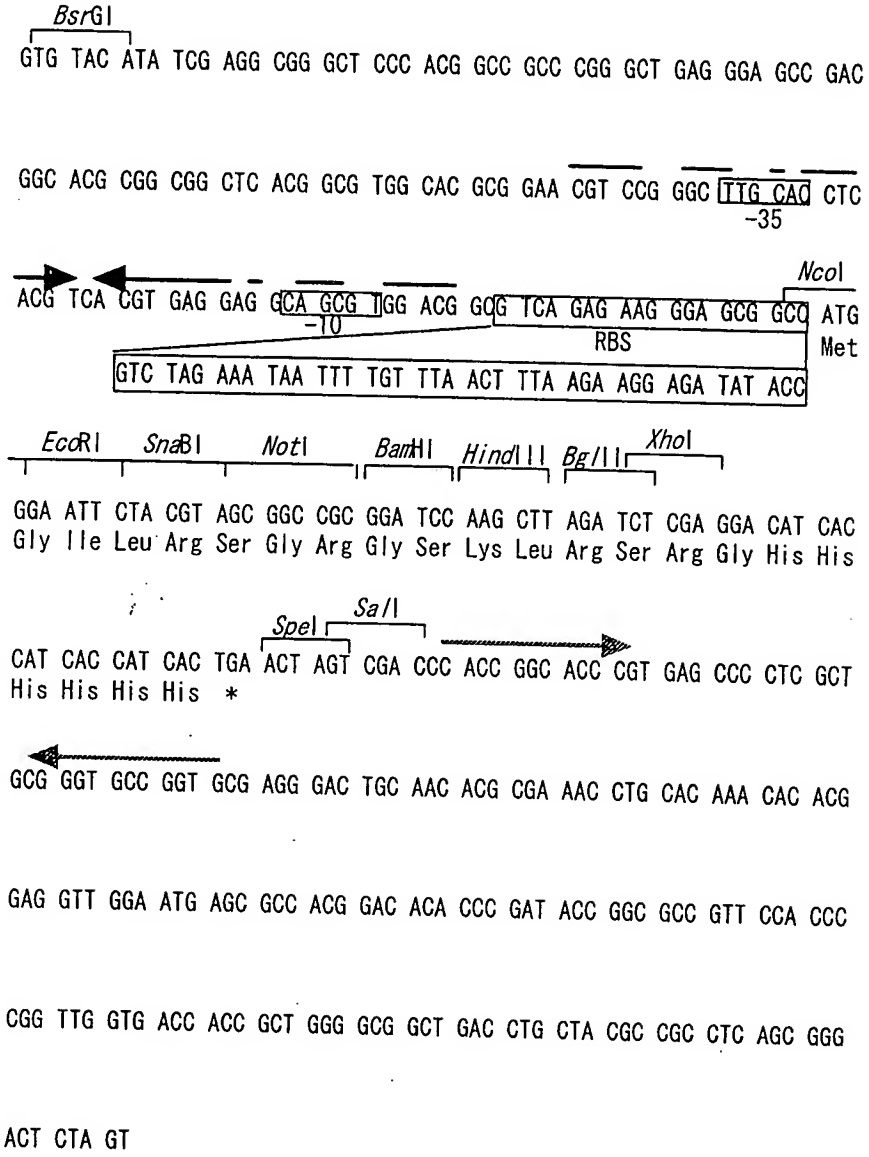
12/31

Fig. 9b



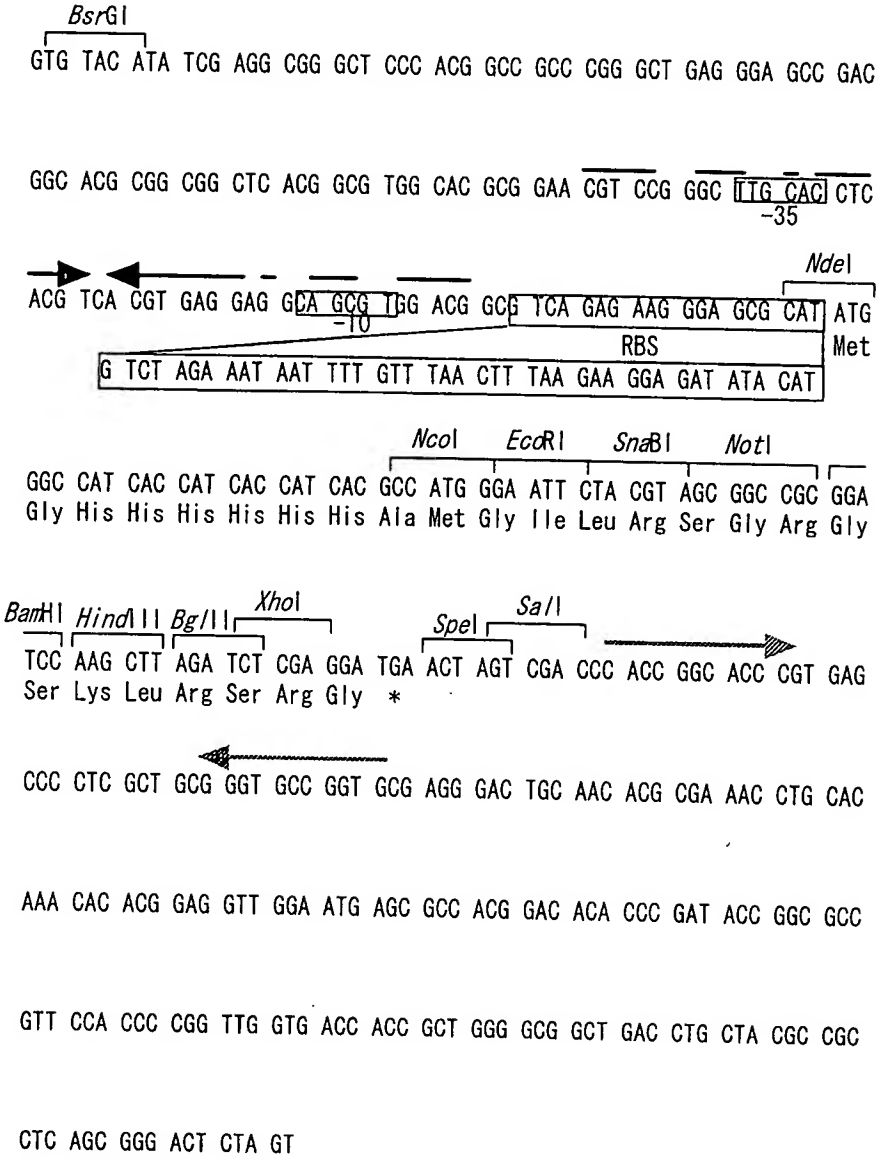
Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232
10/524193

Fig. 9c



Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232
10/524193

Fig. 9d



Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232
10/524193

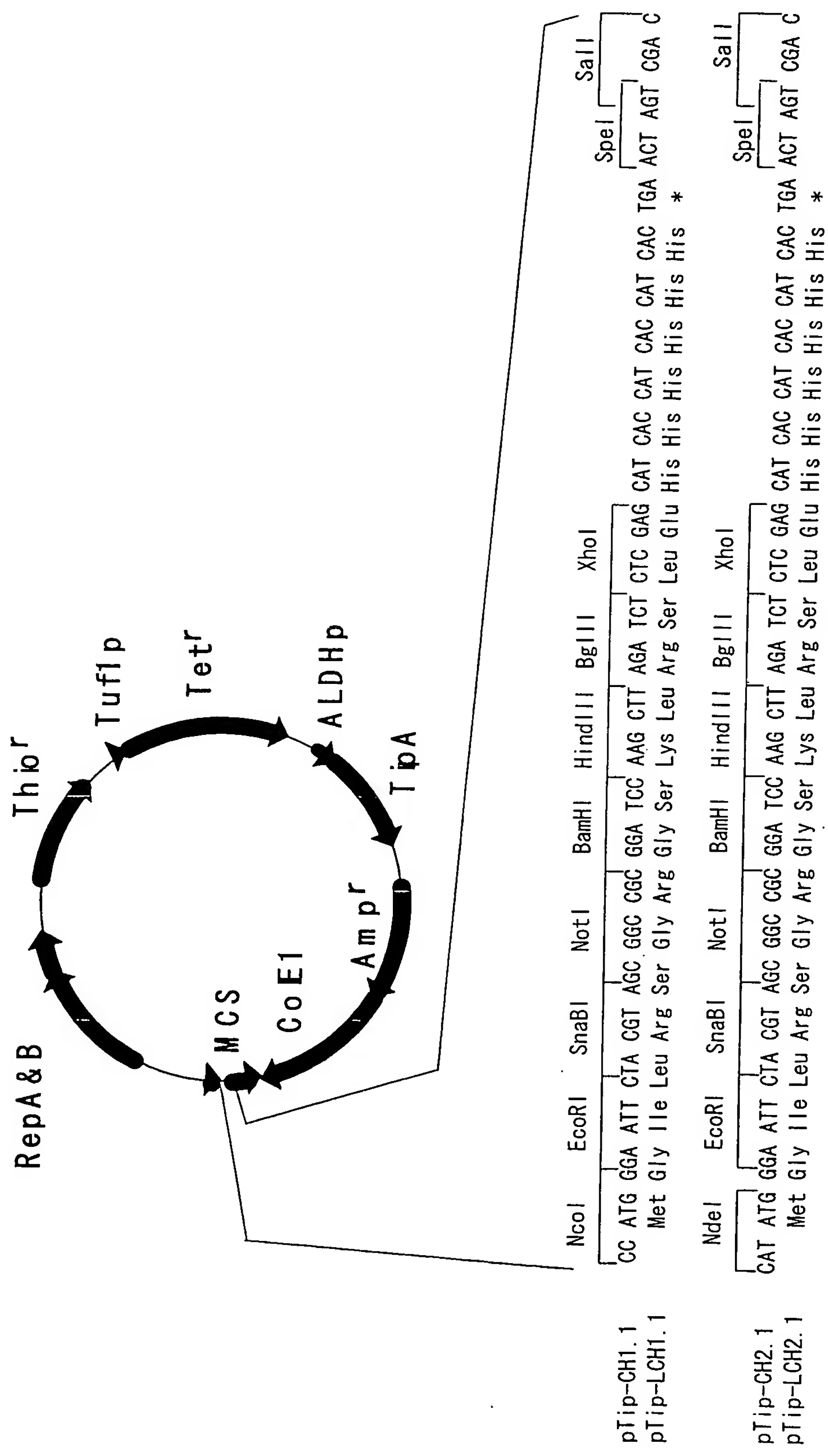
10/524193

Fig. 9e

Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232

10/524193

Fig. 10



10/524193

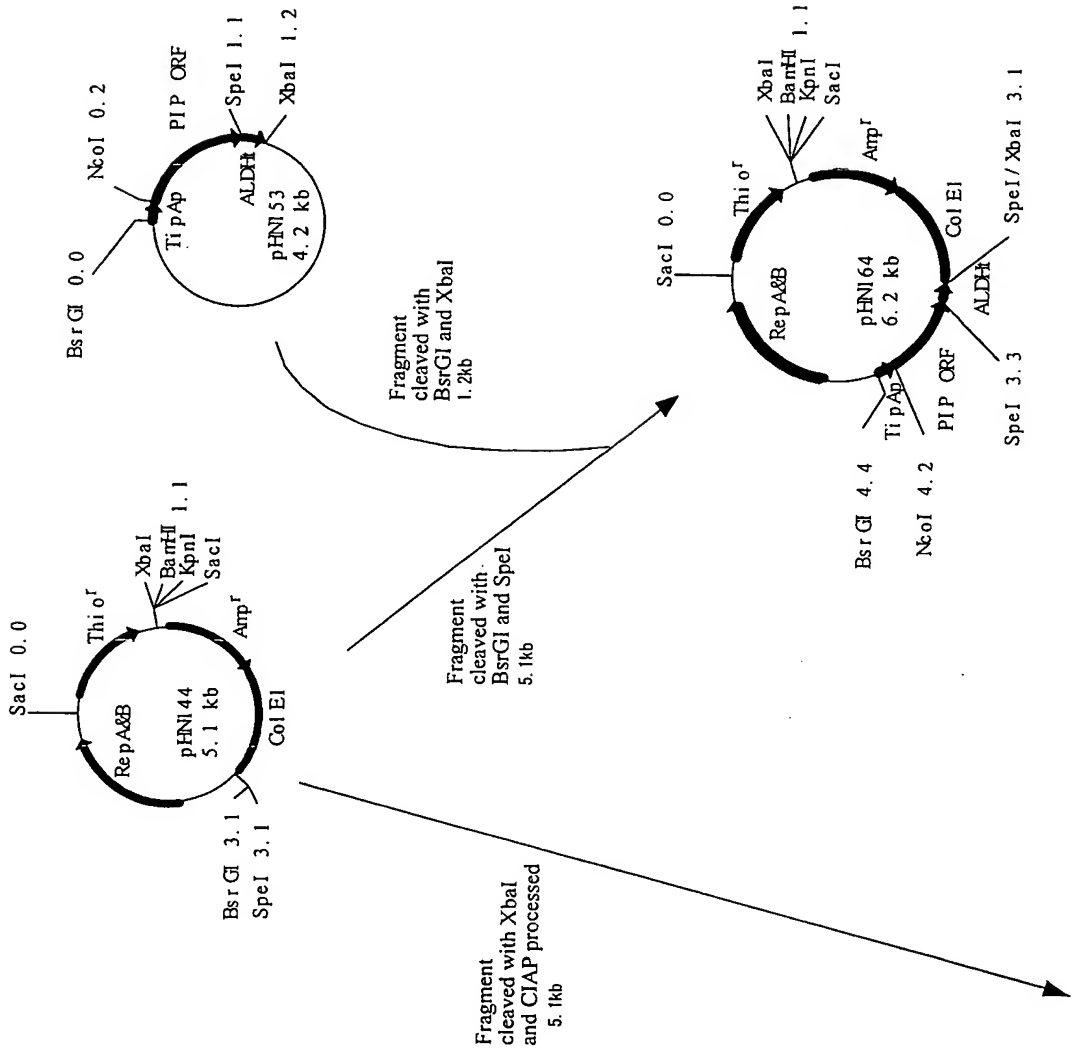


Fig. 11

Fig. 11

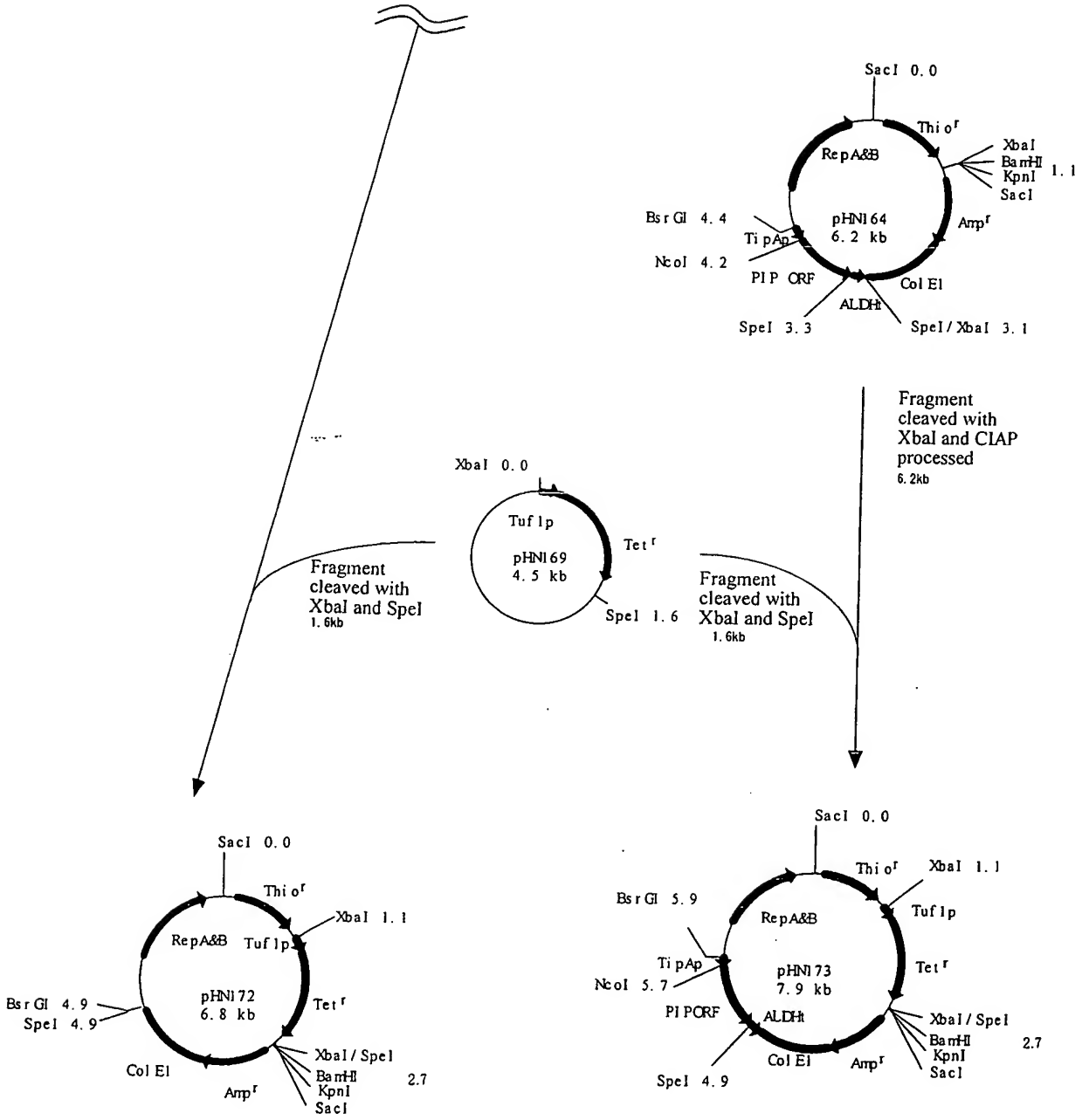
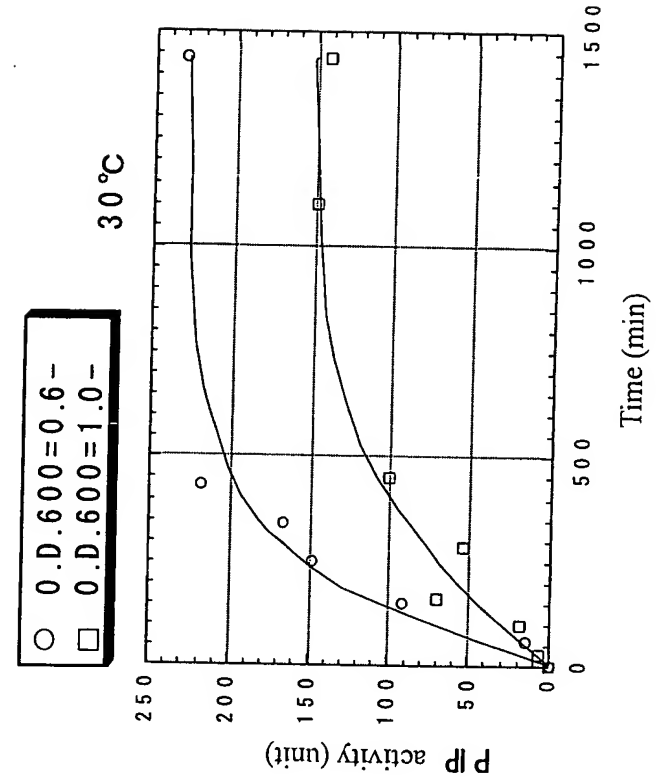

SacI 0.0
Thio^r
RepA&B
XbaI
BamHI
KpnI
SacI
1.1
BsrGI 4.4
TipAp
NcoI 4.2
pHN164
6.2 kb
Amp^r
PIP ORF
ColE1
ALDH
SpeI 3.3
SpeI/XbaI 3.1
XbaI 0.0
Tuf1p
pHN169
4.5 kb
Tet^r
SpeI 1.6
Fragment cleaved with XbaI and SpeI
1.6kb
Fragment cleaved with XbaI and CIAP processed
6.2kb
Fragment cleaved with XbaI and SpeI
1.6kb
SacI 0.0
Thio^r
XbaI 1.1
RepA&B
Tuf1p
pHN172
6.8 kb
Tet^r
BsrGI 4.9
SpeI 4.9
ColE1
Amp^r
XbaI/SpeI
BamHI
KpnI
SacI
2.7
SacI 0.0
Thio^r
XbaI 1.1
BsrGI 5.9
RepA&B
Tuf1p
TipAp
NcoI 5.7
pHN173
7.9 kb
Tet^r
PIPORF
ALDH
XbaI/SpeI
BamHI
KpnI
SacI
2.7
ColE1
Amp^r
SpeI 4.9

Fig. 12

1μg/ml Thiostrepton + −	Activity (+/-Thiostrepton) (unit)	Culturing Temperature (℃)	Culturing volume (μl)	Plasmid used for transformation of *R.erythropolis*	Inducer cassette: ALDHp TipA	Expression cassette: TipAp PIP ORF ALDHt
	16/0.5	4℃	5	pHN170	+	+
	0.1/0.2	4℃	5	pHN173	−	+
	0.1/0.1	4℃	5	pHN172	−	−
	241/4	30℃	0.5	pHN170	+	+
	0.9/0.6	30℃	0.5	pHN173	−	+
	0.3/0.3	30℃	0.5	pHN172	−	−

10/524193
Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232


30 °C
○ O.D.600=0.6
□ O.D.600=1.0
P IP activity (unit)
Time (min)
0
50
100
150
200
250
0
500
1000
1500

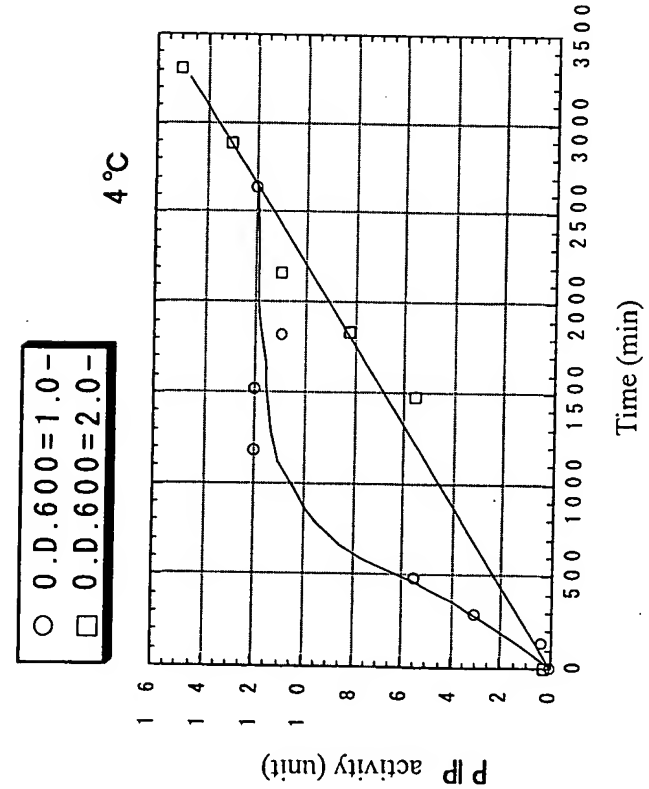
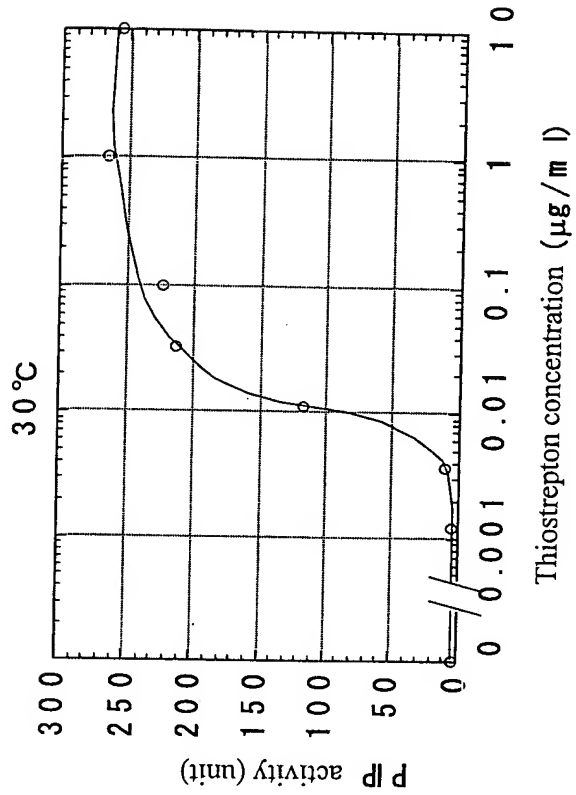

4 °C
○ O.D.600=1.0
□ O.D.600=2.0
P IP activity (unit)
Time (min)
0
2
4
6
8
10
12
14
16
0
500
1000
1500
2000
2500
3000
3500

Fig. 13


30℃
PIP activity (unit)
300
250
200
150
100
50
0
0
0.001
0.01
0.1
1
10
Thiostrepton concentration (μg/ml)

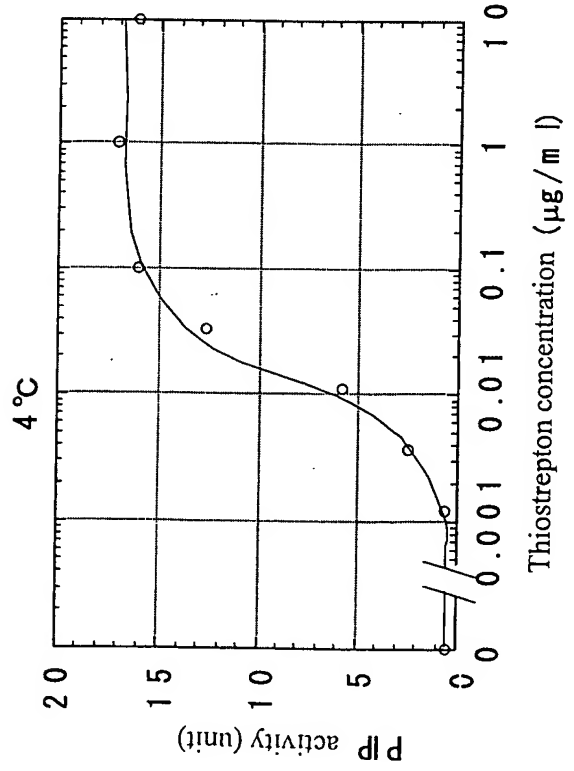
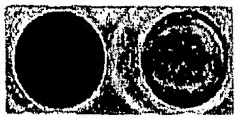
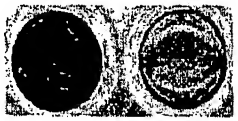
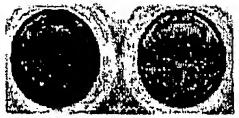
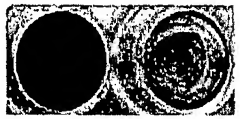
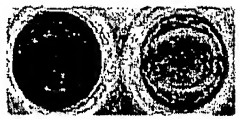
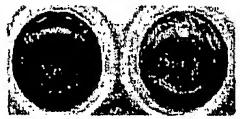

4℃
PIP activity (unit)
20
15
10
5
0
0
0.001
0.01
0.1
1
10
Thiostrepton concentration (μg/ml)

Fig. 14

Fig. 15

1μg/ml Thiostrepton	Activity (+/-Thiostrepton) (unit)	Culturing Temperature (℃)	Host strain transformed with pHN170	Culturing volume (μl)
+ −				
	13/0.8	4℃	*R.erythropolis*	20
	7/0.8	4℃	*R.fascians*	20
	1.9/0.3	4℃	*R.opacus*	100
	215/2	30℃	*R.erythropolis*	2.5
	34/0.4	30℃	*R.fascians*	2.5
	6/1	30℃	*R.opacus*	20

Title: Novel expression vector suitable for expression of recombinant protein at low temperature
Inventors: Nakashima et al.
Atty. Dkt. No.: 081356-0232
10/524193

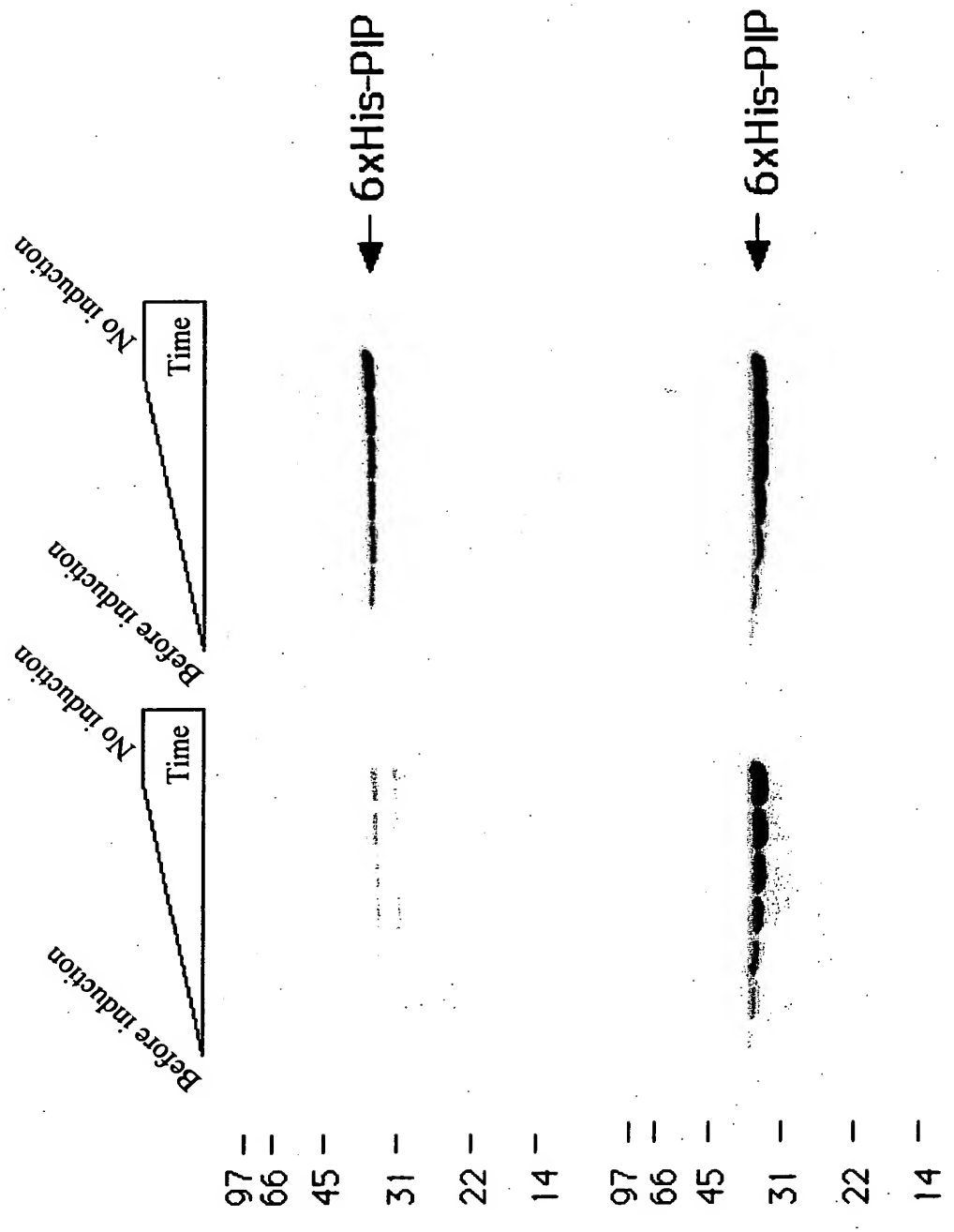

6xHis-PIP
6xHis-PIP
No induction
Time
Before induction
No induction
Time
Before induction
97
66
45
31
22
14
97
66
45
31
22
14
4°C
30°C
TipA promoter
TipA-LG10 promoter

Fig. 16

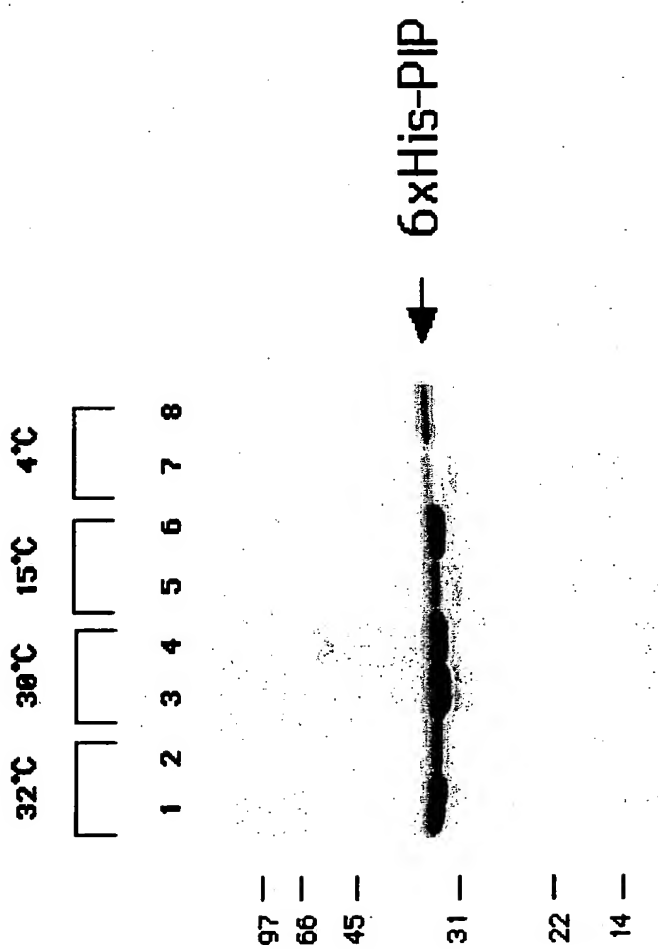


Fig. 17

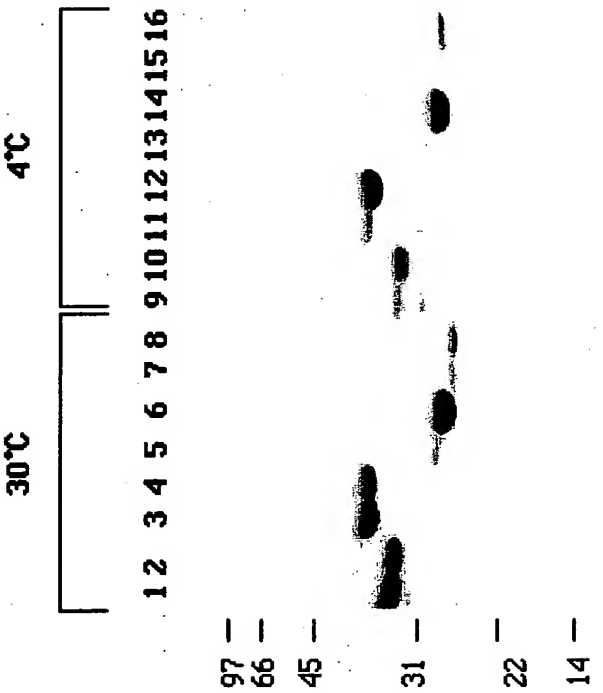


Fig. 18

Fig. 19

Temperature	Reporter	WT	LG10	Magnification (LG10/WT)
30°C	PIP	11	6.3	0.57
	AtPIP	11	4.6	0.39
	GFP	1.1	10	9.1
	GST	0.16	1.3	8.1
4°C	PIP	0.29	2.6	8.9
	AtPIP	0.13	2.9	22
	GFP	<0.01	3.9	>390
	GST	<0.01	1.3	>130

Fig. 20

Name of plasmid	Class	GenBank Accession No.	Type	Number
	Functionally known protein (two or more obtained)		6	16
LE20	Serum amyloid A (Saa1)	M11131		6
L113	NADH dehydorogenase 1 α 4	BC011114		2
LE59	Pantotenate kinase 1 β	AF200357		2
LE94	Retinol binding protein 4 (RBP4)	AK008765		2
LE98	Major urinary protein 4 like	BC019965		2
LE287	Histidine-rich glycoprotein	NM_053176		2
	Functionally unknown protein (singly obtained)		24	24
L3	Cytochrome b5 like	AK002426		1
LE2	Fibrinogen A alpha	BC005467		1
LE3	Clusterin	NM_013492		1
LE9	Splicing factor 3b subunit 1 155kDa	NM_031179		1
LE12	Haptoglobin	NM_017370		1
LE18	Peroxiredoxin 4	BC019578		1
LE82	Inter-alpha-trypsin inhibitor Heavy Chain 2	NM_010582		1
LE87	RIKEN130000F09, Highly similar to VIP36	NM_025828		1
LE95	Serum albumin	AJ011413		1
LE125	Arylacetate deacetylase	BC019999		1
LE137	New cDNA, Highly similar to UDP-Glycosyltransferase	-		1
LE156	RIKEN1300017J02, Highly similar to Transferrin	AK005035		1
LE171	Phosphatidylinositol 3-kinase	NM_008839		1
LE178	Protein kinase C receptor (RACK1) like	D29802		1
LE204	EGF receptor	AF275367		1
LE247	Retinoic acid receptor responsive protein TIG2	AK002298		1
LE251	Insulin-like growth factor IA	X04480		1
LE280	Transferrin	BC022986		1
LE295	Apolipoprotein A-V	NM_080434		1
LE305	Fatty Acid Binding Protein 1 (FABP1)	BC009812		1
LE354	Retinoblastoma binding protein 7 (Rbbp7)	NM_009031		1
LE357	Zinc fingers and homeoboxes protein 1 (Zhx1)	NM_009572		1
LE416	Tumor differentially expressed 1 like (Tde1l)	NM_019760		1
LE421	RIKEN1300006C19, Highly similar to OSTSTT3	AK018758		1
	Functionally unknown protein		4	4
LE25	IMAGE:4239007, DUF92 like membrane Protein?	BC016895		1
LE51	New cDNA, No homology	-		1
LE119	IMAGE:3489640, Bone marrow stromal protein like?	BC008532		1
LE123	RIKEN1500015G18, No homology	NM_025439		1
	Subtotal		34	44
	Other proteins (out of ORF or not important proteins)			382
	Total			426

Fig. 21

category	Protein	6xHis tag	Presumed molecular weight	*R. erythropolis* Name of plasmid	30 °C Sup/Ppt	30 °C Proliferation	4 °C Sup/Ppt	*E. coli* Name of plasmid	30 °C Sup/Ppt	30 °C Proliferation
Protein isolated by screening	Saa1	N'	12 (14)	pHN205	0.4/1	−	0.08/2	pHN193	N.D./N.D.	+−
	NADH4	N'	9	pHN206	N.D./0.2	+	N.D./0.2	pHN195	N.D./N.D.	++
	Cytochrome b5I	N'	15	pHN208	0.2/8	+−	0.5/4	pHN199	N.D./0.8	+
	LE123	N'	19 (21)	pHN287	0.04/0.08	+	0.03/0.06	pHN276	N.D./N.D.	+−
	Transferrin	N'	75 (77)	pHN289	0.2/0.5	+	0.06/0.2	pHN277	0.2/0.2	++
	Apoa5	N'	39 (41)	pHN288	3/8	+−	2/4	pHN281	N.D./N.D.	+++
	PanK	N'	42					pHN279	2/N.D.	++
	Peroxredoxin4	N'	27 (31)					pHN278	4/0.4	++
	TFL	N'	75 (77)					pHN280	0.2/0.2	++
Insoluble protease	Cathepsin D	C'	43 (45)	pHN270	2/3	++	0.3/2	pHN273	N.D./N.D.	+++
	Prothrombin	C'	30 (70)	pHN271	N.D./N.D.	+−	N.D./N.D.			
	Kallikrein6	C'	26 (29)	pHN272	0.3/0.3	+++	0.3/2	pHN275	N.D./N.D.	+++
DNase	LSDNAse	N'	36 (33)	pHN299	N.D./N.D.	+	N.D./N.D.			
	DLAD	N'	38 (41)	pHN284	N.D./N.D.	+−	N.D./N.D.			
Protein inhibiting cell proliferation	HMG-1	N'	25	pHN285	4/0.2	−	2/0.06	pHN305	0.2/0.1	−
	Kid1	N'	66	pHN286	N.D./0.08	−	N.D./0.2			
	Bax alpha	N'	21	pHN217	N.D./N.D.	−	N.D./N.D.	pHN212	N.D./N.D.	−
Protein solubilized with low temperature dependence	Glucokinase	N'	52	pHN298	4/2	++	6/2	pHN306	5/1	+++
	p37A	C'	38	pHN291	4/0.2	+++	3/0.1	pHN308	4/N.D.	+++
Positive control	PIP	C'	33	pHN171	6/0.7	+++	3/0.3			
	LacZ	N'	120					pBAD/HisA/lacZ	4/0.5	+++

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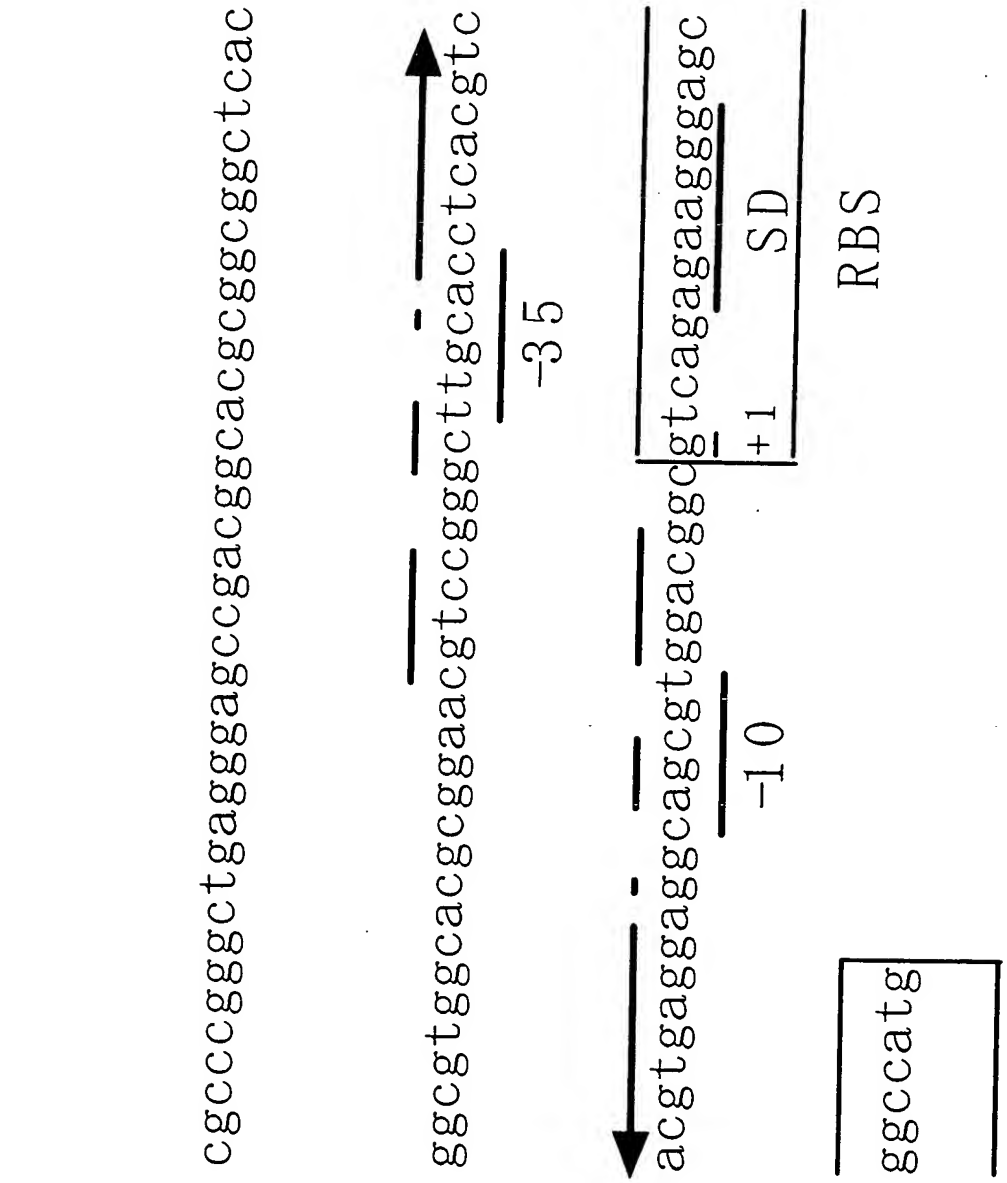
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ggcgtggcacgcggaacgtccgggcttgcacctcacgtc
-35
acgtgaggaggcagcgtggacgggcgtcagaagggagc
-10
+1
SD
RBS
ggccatg

Fig. 22


WT RBS
g tca ggg agg gga ggg gcc atg
SD
LG10 RBS
gtc tag aaa taa ttt tgt tta act tta agg agg tat acc atg
SD

Fig. 23

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